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International Journal of Current Research
Vol. 8, Issue, 11, pp.42200-42205, November, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PARTIAL PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE FROM HALOPHILIC BACTERIA *Bacillus* sp

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ARTICLE INFO

Article History:
Received 08th August, 2016
Received in revised form
20th September, 2016
Accepted 21st October, 2016
Published online 30th November, 2016

Key words:

Alkaline Protease,
Characterization,
Halophilic bacteria,
Metal ions,
Purification.

ABSTRACT

Alkaline protease, a hydrolytic enzyme from a novel strain of halophilic bacteria *Bacillus* sp. was purified up to 1.42 fold purification with a recovery of about 5% and then characterized at various operating conditions. Zymography of the different fractions showed the hydrolysis of casein by the enzyme. The maximum activity of purified alkaline protease was observed at pH 9.0 and at a temperature of 50°C using casein as substrate, respectively. Certain metal ions like $MgCl_2$, $CaCl_2$ andβ-mercaptoethanol enhanced alkaline protease activity while $HgCl_2$and EDTA inhibited the enzyme activity. TheKm and Vmax for partially purified enzyme were 1.17mg/ml and 2.22mg/ml/min respectively.

Citation: Khushbu Kanth, TesnimArfi and Vinod Kumar Nigam, 2016. "Partial purification and characterization of alkaline protease from halophilic bacteria bacillus sp", *International Journal of Current Research*, 8, (11), 42200-42205.

INTRODUCTION

Alkaline protease is a hydrolytic enzyme which hydrolyses a number of proteinaceous substrates. It occurs in plants, animals and in microorganisms. Microbial alkaline proteases are produced by a number of microorganisms including yeasts, moulds and bacteria. It is mostly secreted as an extracellular enzyme. (Balakrishnan *et al.*, 2012) Halophiles are a type of extremophiles that survive in extreme environmental conditions. These microbes are reported to produce a number of novel compounds of commercial interest. Alkaline protease is very important industrial enzymes, when compared to other types of proteases. Bacterial alkaline protease with high activity and stability in alkaline range are useful in various industrial applications like in leather industries, food industries, pharmaceutical industries, textile industries, biofilm degradation, silver recovery from used x-ray films and also in detergent industries as detergent additives. Alkaline protease accounted for 40% of the total worldwide enzymes sales (Ellaiah *et al*., 2002; Prakasham *et al*., 2006). These are also used for cleaning of membranes used in protein ultrafiltration.

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Since, halophilic enzymes have adjusted to harsh environments, they are usually stable and therefore, these proteins could serve asa suitable candidate for industrial process which is performed under extreme situations (Joshi *et al*., 2007; Singhal *et al.*, 2012; Nigam *et al.*, 2013a; Dixit and Nigam, 2014). The purification and characterization is the basic need to elucidate the various properties of enzymes. The purification and characterization of alkaline proteases from the halophilic sources have been gaining momentum these days because of certain varied properties of enzyme and therefore, an attempt has been taken to purify the alkaline protease from the halophilic bacteria *Bacillus* sp. followed by study of its kinetic properties.

MATERIALS AND METHODS

Bacterial strain and culture conditions

A halophilicalkaline protease producing bacteria *Bacillus* sp.was used for the production of enzyme in 12% modified growth medium (MGM) (Nigam *et al*., 2013b).The enzyme production medium consisted of: $MgSo_4$ 14g/L, $Mgcl_2$ 12g/L, NaCl 120g/L, KCl 2.8g/L, $CaCl_2$0.55g/L, yeast extract 1g/L and casein 5g/L. The pH of production medium was adjusted to 8.0 before sterilization.

The alkaline protease enzyme from the selected microorganism was cultivated as batch mode for 72 h at 37 ºC under an agitation speed of 175 rpm.

Enzyme Assay

After completion of growth, the production broth was centrifuged at 10000 rpm under refrigeration for 10 minutes. The supernatant was used to test alkaline protease activity. Alkaline protease activity is measured by a method described by Makhija *et al*, (2006). 1ml of 1% casein in 0.1M tris-HCl buffer (pH-9.0) was incubated with diluted crude alkaline protease at 50°C for 30 minutes and the enzyme-substrate reaction was stopped by adding 0.5ml of 10% trichloroacetic acid (TCA) and further centrifuged at 10000 rpm for 10 minutes to collect the supernatant for tyrosine estimation at 660 nm. A control reaction was also prepared where TCA was added before incubation. One unit of alkaline protease activity (U) is defined as µg of tyrosine released per ml per minute under specified conditions of assay.

ENZYME PURIFICATION

Ammonium sulfate precipitation

Ammonium sulfate salt was used to precipitate enzyme protein by increasing the concentration of salt. Fractional precipitation was performed to achieve the solubility of desired protein at a particular concentration by the mentioned formula. % of salt saturation = $533 \times (S_2\text{-}S_1)/100 - (0.3\times S_2)$ where, S_1 and S_2 are initial and final concentration of ammonium sulphate salt respectively. The supernatant obtained was fractionated by precipitation with ammonium sulphate up to 75% saturation. Precipitated proteins were separated by centrifugation at 10000 rpm for 10minutes at 4°C and dissolved in tris-HCl buffer of pH 9.

Dialysis: Dialysis of different protein fractions were carried out in dialysis bags (MWCO-10KDa) for 8-9h against buffer. The buffer was changed every 3h. After dialysis, the dialyzed protein was removed from dialysis bag and enzyme assay was performed to confirm alkaline protease protein activity.

Protein estimation: The protein was estimated by the Lowry method using bovine serum albumin as standard at 750nm (Lowry*et al*., 1951).

Column chromatography: The size exclusion chromatography was used for separation and purification of desired protein from the mixture of different proteins. A column (10 cm x 1 cm) was prepared where sephadex G- 25 was used as stationary phase. The column was washed with buffer for 3-4 elution volume. 1 ml of desired fraction of protein was loaded to the column and eluted with the tris-HCl buffer. About 15 fractions were collected and measured for protein content and enzyme activity respectively.

CHARACTERIZATION OF PARTIALLY PURIFIED ALKALINE PROTEASE

Effect of temperature on enzyme activity and stability: The influence of different temperatures on the activity of alkaline protease was determined by standard protocol at various temperatures ranging from 40º to 70º C and relative activity was calculated.

Effect of pH on the activity of purified alkaline protease: The partially purified enzyme was incubated in presence of substrate dissolved in buffers of different pH at 50 ºC for 30 minutes. The buffers used were citrate pH-4 and 5, phosphate buffer pH-6 to 8, tris-HCL pH- 9, borate buffer pH-10 and 11. The alkaline protease activity was finally calculated.

Thermal stability of alkaline protease

Thermostability of alkaline protease was studied at optimum temperature for different time intervals like 0h, 1/2h, 1h, 2h, 3h, 4h, 5h, and 6h respectively and relative activity was determined.

Effect of different substrates on the activity of purified alkaline protease

Three different substrates such as casein, gelatine and keratin at 10g/L concentration, respectively were used for estimation of alkaline proteases activity by purified enzyme secreted from *Bacillus* sp. at standard assay conditions and activity was presented.

Effect of different metal ions on the activity of purified alkaline protease

The alkaline protease was incubated with various metal ions at a final concentration of 5mM to observe their effect on enzyme catalysis. $FeSO_4.7H_2O$, $CaCl_2$, $CoCl_2$, $HgCl_2$, $MgCl_2.6H_2O$, $CuCl_2$, $AgNO_3$, $ZnSO_4.7H_2O$, $NiCl_2.6H_2O$, EDTA and Beta-mercapto ethanol were used in enzyme substrate reaction at operating condition and later the activity was computed against control. All the experiments were performed in triplicates and mean value was reported.

Effect of substrate concentration on activity of alkaline protease

The partially purified alkaline protease activity was estimated in presence of different concentrations of substrate casein at optimal conditions and Km and Vmax were calculated.

Zymography

The zymography of purified protease was carried out by non-reducing substrate PAGE, using 5% (w/v) stacking gel and 10% (w/v) separating gel, with 0.05% (w/v) casein incorporated in the separating gel. After electrophoresis, the gel was washed twice with tris HCL buffer of pH 8.5 containing 2.5% (v/v) Triton X-100. Further washing was performed to remove the Triton X-100.The gel was placed in the same buffer in presence of 0.2 M NaCland 5 mM $CaCl_2$ at 50 °C for 3 h and later stained with 0.25% (w/v) Coomasie Brilliant Blue R-250 overnight for observing the hydrolysis of casein as clear bands. The clear bands against blue background indicated the presence of proteolytic activity of the enzyme (Thangam and Rajkumar, 2002).

RESULTS

*Bacillus*sp was grown at optimal conditions in presence of 12% sodium chloride and alkaline protease activity was calculated against standard plot of tyrosine (Figure 1). The calculated crude activity was 36.8 U.

Purification of alkaline protease: The purification of alkaline protease was initiated by ammonium sulfate precipitation using different saturation concentrations of salt followed by dialysis against buffer for 6-8h. Different saturations by salt were attempted by varying the concentration of ammonium sulfate followed by dialysis. The alkaline protease activity along with concentration of protein achieved during purification process was presented in Table 1. It has been noted that 50-75% saturated protein showed maximum activity and used for further purification purposes of enzyme.

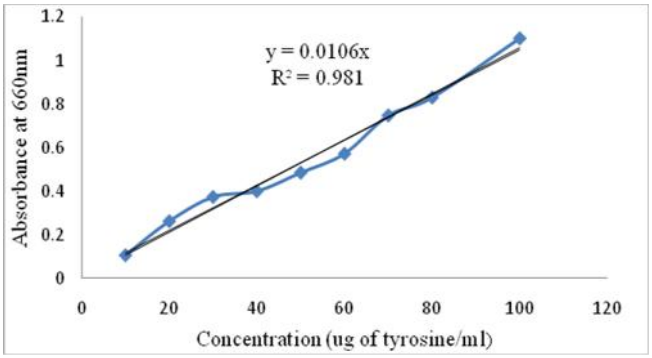

Fig. 1. Standard plot of Tyrosine

Table 1. Alkaline protease activity of salt precipitated proteins at different saturations

S. No.	Fractions of dialyzed enzyme	Total protein (mg)	Activity (U)
1.	0 - 20 %	0.0009	0.32
2.	20 - 50 %	0.0032	2.82
3.	50 – 80 %	0.0044	5.33
4.	0 -40 %	0.0011	0.42
5.	40 – 80 %	0.0026	4.80
6.	0 -30 %	0.0016	0.26
7.	30 – 60 %	0.0034	2.0
8.	60 – 80 %	0.0046	2.5
9.	0 - 30 %	0.0013	0.37
10.	30 – 50 %	0.0037	15.84
11.	50 – 75 %	0.0055	17.04

The fraction with maximum activity was then purified using column chromatography using a column packed with sephadex G-25. 1ml protein fraction (activity 17.04 U) was eluted using buffer and the activity of certain fractions with positive activity is shown in Figure 2.

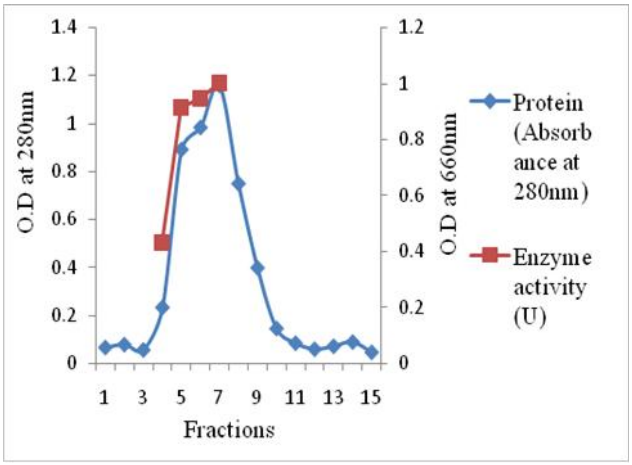

Fig 2. Measurement of protein and alkaline protease activity during column chromatography

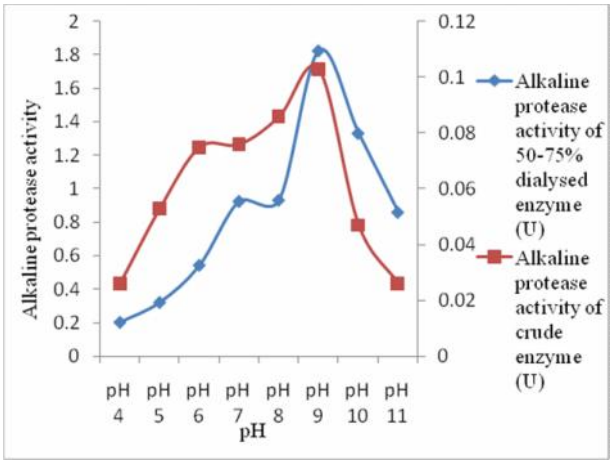

Fig 3. Effect of different pH on purified alkaline protease activity

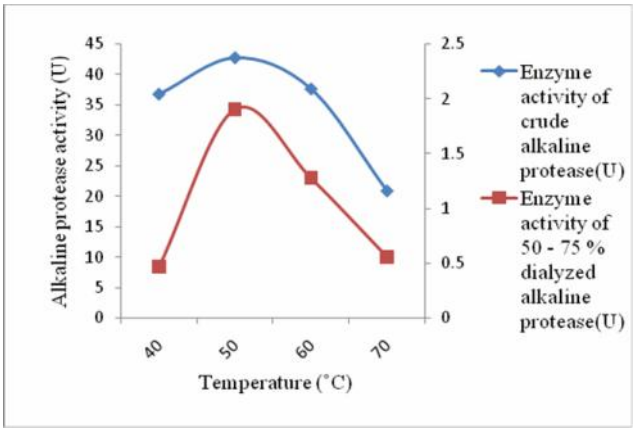

Fig 4. Effect of different temperatures on purified alkaline protease activity

Characterization of partially purified alkaline protease: The purified enzyme was characterized for various kinetic parameters like pH, temperature, stability, substrates and role of metal ions on catalysis etc. Figure 3 illustrates the effect of pH on the alkaline protease activity and observed that a pH of 9 is more suitable for both crude and purified enzyme though the activity of purified enzyme is low. Similarly, for maximum activity at various temperatures a temperature of 50^{o}C was optimum for enzyme catalysis by purified enzyme (Figure 4). Figure 5 describes the effect of different substrates on alkalineprotease activity and observed casein as most preferred substrate for the partially purified enzyme by *Bacillus* sp. The effect of different metal ions on purified alkaline protease activity showed that enzyme catalysis was enhanced by $MgCl_2$, $CoCl_2$, $NiCl_2$and $CaCl_2$while,β-mercaptoethanol, EDTA and $HgCl_2$inhibited the activity(Figure 6). Thermostability of crude and purified enzyme was also studied (Figure 7) and found that half life of purified enzyme is less than two hours at standard conditions as compared to crude enzyme (half life - more than 5 hours).The effect of varying concentration of casein on enzyme substrate reaction was studied for evaluation of MachelisMenten Constant (Km) and maximum reaction velocity (Vmax)using double reciprocal plot of 1/Sagainst 1/V (Figure 8).TheKm and Vmax for partially purified enzyme were 1.17mg/ml and 2.22mg/ml/min respectively.

Zymography of partially purified alkaline protease: The activity of partially purified alkaline protease was observed by Zymography.

Table 2. Summary of purification of alkaline protease

S. No	Samples	Total protein (mg)	Activity (U)	Specific activity (U/mg)	Fold purification	Recovery (%)
1.	Crude (15ml))	0.68	148	14.5	1	100
2.	Amicon sample (10ml)	0.72	144	20	1.2	97
3.	50 – 75 % (3.0ml)	0.83	84	24	1.42	56
4.	Column chromatography (2ml)	0.86	8.5	-	-	4.9

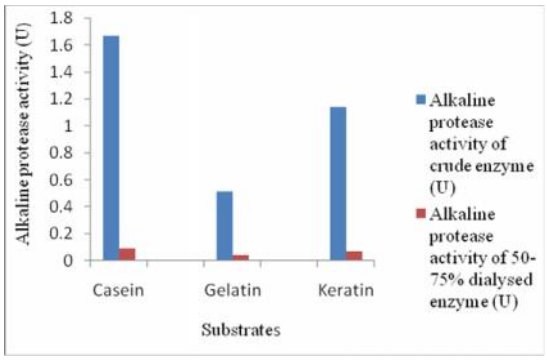

Fig. 5. Substrate specificity of purified alkaline protease

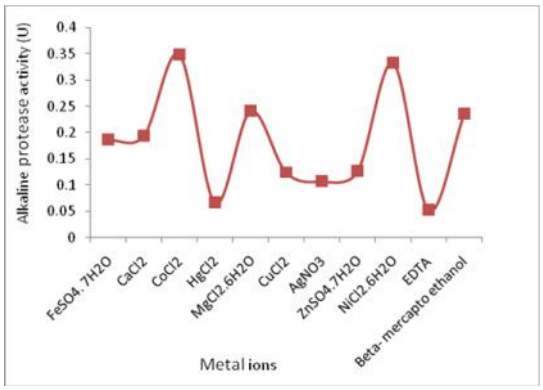

Fig. 6. Effects of metal ions on puriedalkaline protease activity

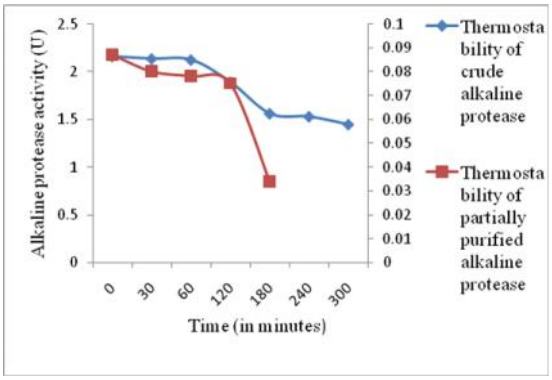

Fig 7. Effect of temperature on the stability of alkaline protease at 50˚C

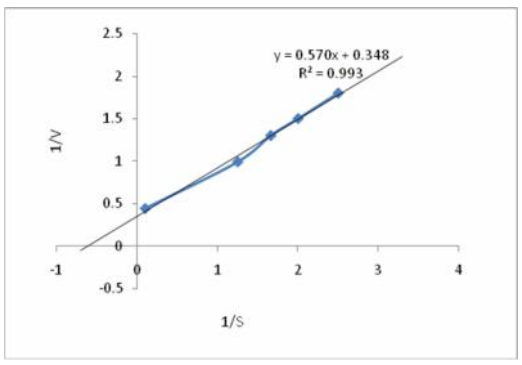

Fig. 8. Double reciprocal plot (1/S versus 1/V)

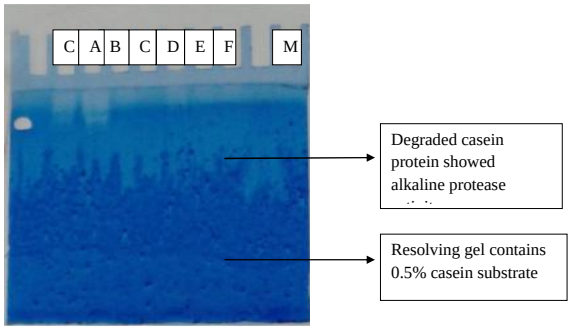

Fig 9 Zymography: M-marker protein, C- crude enzyme, A-concentrated alkaline protease, B & C – saturated by ammonium sulfate precipitation, D, E & F are fractions of partially purified alkaline protease

The gel containing casein as substrate was partially digested by crude enzyme lane (C) and also by concentrated enzyme lane (A). It was completely digested by purified enzyme fractions, lane (D, E,F). Table 2 summarizes purification processes used for alkaline protease produced extracellualrly from a halophilic mocroorganim*Bacillus* sp. It has been observed that maximum recovery was observed at 50-75% saturation of protein by ammonium salt but drastically decresed during column chromatography.

DISCUSSION

Bacillus sp. is the most important organism for commercial alkaline protease production due to their high thermo stability and pH stability. The production of alkaline protease from most of the bacterial strains observed in the range of 2-4 days of incubation period (Shumi *et al.*, 2004; Akcan *et al*., 2011; Kumar *et al*., 2012). In the present study the highest alkaline protease specific activity of 14.5U/ml was observed at 72h of fermentation period. The maximum alkaline protease production is noticed in production media of pH ranges between7-13 (Pouryafar *et al*., 2015). The present investigation also showed that *Bacillus* sp. produced maximum alkaline protease at pH 9 and it was stable at pH 9. The alkaline protease is relatively stable in the temperature range 50-70˚C (Laxmi *et al*., 2015; Tremacodi *et al.*, 2007). Studies has been performed on halophilic bacteria and the purification of enzyme from halophilic sources is difficult due to presence of high concentration of salt. In this study, fractional saturation was carried out by ammonium sulfate precipitation and the alkaline protease activity was monitoredat every fractions of precipitated proteins (Table 1). The dialysed 50-75% saturated fraction showed maximum alkaine protease activity and it was further taken for purification. The fraction 6, 7 & 8 from column chromatography showed maximum alkaline protease activity (26.4U, 30U & 27.7U) respectively (Fig. 2). In a reported paper, 10% purified alkaline protease enzyme was recovered from *Bacillus licheniformis* by performing DEAE cellulose chromatography followed by gel filteration using Sephadex G-100 (Lakshmi *et al.*, 2015). In another reported paper, the enzyme was purified using ammonium sulphate precipitation with 2.64 fold purification at 70% of ammonium salt saturation (Mukhtar*et al.*, 2012).

The present result showed 1.42 fold purification with 75% saturation with 5% of recovery (Table 2). Most of the purified alkaline protease showed a temperature range for caseinolysisin between 50 to 70˚C (Lakshmi *et al.,* 2015; Balakrishnan *et al.,* 2012; Sharma *et al.,* 2014; Nadeem *et al.,* 2013; Tremacoldi *et al.,* 2007; Banik *et al.,* 2006). One species of *Bacillus* showed 40˚C as optimum temperature for maximumactivity (Agrawal *et al.,* 2012). The present investigation showed high alkaline protease activivity of purified protein at temperature at 50˚C (Fig. 4). The optimal pH for purifird protease enzyme was reported in pH range of 8-11 (Balakrishnan *et al.,* 2012; Sharma *et al.,* 2014; Agrawal *et al.,* 2012; Nadeem *et al*., 2013), while in current study, the purified enzyme showed pH 9 as most optimum for maximum activity (Fig 3). Pathak *et al.* (2012) showed the enzyme production in presence of casein (10g/L) while in this work alkaline protease production occurred at 5g/L of casein. The enzyme from *Bacillus licheniformis* UV-9 showed substrate preference as casein from various proteinaceous substrates like Casein, haemoglobin, keratin, collagen and albumin (bovine) (Nadeem *et al.,* 2013). In contrast, this study revealed three proteinaceous substrate were hydrolysed by purified alkaline protease though the activity was higher for casein (Fig 5). In another study, the alkaline protease activity was greatly reduced in the presence of $MnCl_2$, whereas $MgCl_2$ enhanced its activity (Mukhtar *et al.,* 2012) while present investigation showed among all metal ions, $MgCl_2$, $CoCl_2$, $NiCl_2$& $CaCl_2$ enhanced alkaline protease activity and it was inhibited by β-mercaptoethanol, EDTA& $HgCl_2$ (Fig 6).The Km value of 1.17 mg/ml and Vmax of 2.22mg/ml/min was observed in the presnt work by puried enzyme of halophilic *Bacillus* which indicates relatively low Km as compared with other reported study. Though most of the comparisons were made from non-halophilic sources of alkaline protease and thus this work provides a platform for purification of enzyme from various extemophiles.

Conclusion

An attempt has been taken for purifying the protease from halophilic *Bacillus* sp.with a recovery of about 5%. The partially purified enzyme was stable at alkaline pH of 9 and temperature of 50°C. Certain divalent ions enhanced protease catalysis while purified enzyme activity was inhibited by β-mercaptoethanol, $HgCl_2$ and EDTA. The enzyme showed capability of hydrolysing casein, keratin and gelatine at high temperature. The Thermostability of purified alkaline protease was checked up to 5h at 50 ^oC and it was stable up to 2h in alkaline pH range. One associated problem during the purification steps was to remove salt (12% sodium chloride) as *Bacillus* sp produced maximum enzyme at this concentration.

Aknowledgement

I greatly acknowledge Dr. V. K. Nigam Associate professor of BIT Mesra for guidance and support to carry out this research. I would also like to convey special thanks to Ms. TesnimArfi for inspiring enthusiasm and generosity in sharing her knowledge in this field.

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